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14. ABSTRACT

Currently available therapies for breast cancer are limited by nonspecificity and system toxicities. Nanotechnology-based approaches offer an elegant means to overcome these limitations by multiplexing functional chemistries for medical image contrast and therapy with moieties for enhanced solubility and drug delivery. The dually-targeted, multifunctional nanoparticles we are developing are designed to selectively target and effectively diagnose or treat breast tumors, limiting damage to normal tissue and reducing side effects associated with traditional cancer therapies. The findings reported herein demonstrate that we have the ability to synthesize and characterize the key components of protelytic nanobeacons (PNBs) and proteolytic nanotherapeutics (PNTs). Furthermore, we have verified enzymatic cleavability and of substrate peptides and established methods by which to assess MMP selectivity. Evidence from in vitro and in vivo experiments suggests that the proposed strategy for MMP-activated selective delivery of diagnostic and therapeutic reagents is feasible. We propose to proceed with the development of PNBs and PNTs with broadly MMP-selective substrates in parallel with screening for one appropriate for MMP14. The ultimate goal of this research is to provide a means to improve both the survival and the quality of life of people diagnosed with breast cancer.

15. SUBJECT TERMS
breast cancer, invasion, metastasis, invadopodia, nanobeacons, dendrimer, mmp, mmp-14, mt1-mmp, folate, folate-targeting

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INTRODUCTION

Important clinical priorities in the battle against breast cancer include improved detection and prognosis of early stage malignancies as well as rapid development of targeted therapies which preferentially deliver diagnostic and therapeutic agents to tumor tissues while sparing normal ones. Limitations of anti-cancer treatments currently available in the clinic include inefficacious delivery, nonspecific injury to normal human cells due to nonselective cellular uptake, and in some cases both. In many instances, less than one percent of the injected dose of an administered chemotherapeutic agent can be found in tumor cells. To address this concern, the presented research aims to develop and test novel nanoparticles which combine both enzymatic activation by matrix metalloproteinases (MMPs) and molecular targeting by folic acid attachment with other functionalities to achieve selective delivery of diagnostic and therapeutic reagents to breast tumor cells.

BODY

The first specific aim of this research is to chemically synthesize and characterize the proposed nanoscopic macromolecules deemed proteolytic nanobeacons (PNBs) and proteolytic nanotherapeutics (PNTs) and to assess proteolytic cleavage of them in cell-free systems and in vitro. The aspects of this work which we have completed according to the proposed *Statement of Work* are presented herein:

Task 1: Synthesize and characterize PNBs and PNTs, verify MT1-MMP-selective cleavage, and assess in-vitro proteolysis of PNBs and PNTs.

a) PNBs: Attach FITC-conjugated MT1-MMP-selective substrate peptides, tetramethylrhodamine (TAMRA; reference dye and FRET quencher) and PEG-1000 to commercially available PAMAM-3 dendrimer with established chemistries. Validate synthesis with analytical chemistry techniques: UV-vis and fluorescence spectroscopy, HPLC, MALDI-TOF-MS, ninhydrin assay, dynamic light scattering (DLS).

A principally unique component of these nanoparticles is the MMP substrate peptide which forms the basis of the cleavable attachment of the relevant imaging and therapeutic species. We have completed several iterations of assessment and gradual improvement of the synthetic approach to conjugation of fluorescent dye molecules to such peptides for molecular imaging. Notably, we have realized an alternate strategy for the attachment of the fluorophore intended for both tracking of the polyamidoamine (PAMAM) core and for quenching of a donor (sensor) fluorophore by Förster Resonance Energy Transfer (FRET). This fluorescent dye molecule is attached directly to an amino acid of the peptide on the core-facing side of its MMP cleavage site rather than directly to the PAMAM core itself, as previously proposed (Fig. 1). The change facilitates improved FRET interaction by decreasing intermolecular distance and introducing less variation in FRET quenching efficiency.

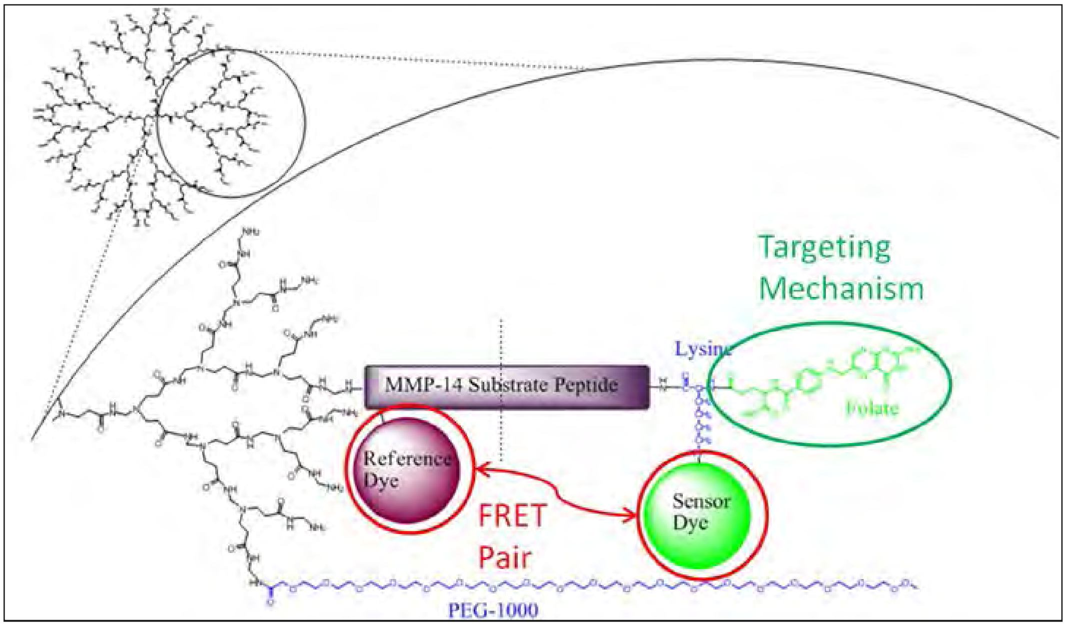


Figure 1: Schematic of PNB reflecting the updated reference dye attachment strategy. The reference dye is covalently linked to the substrate peptide, whereas the previous strategy proposed attachment to the PAMAM core molecule.

Using the revised approach to the synthesis, we attached FRET-paired fluorophores to substrate peptides. We verified this attachment by ultraviolet and visible (UV/Vis) light absorbance (Fig. 2) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Fig. 3).

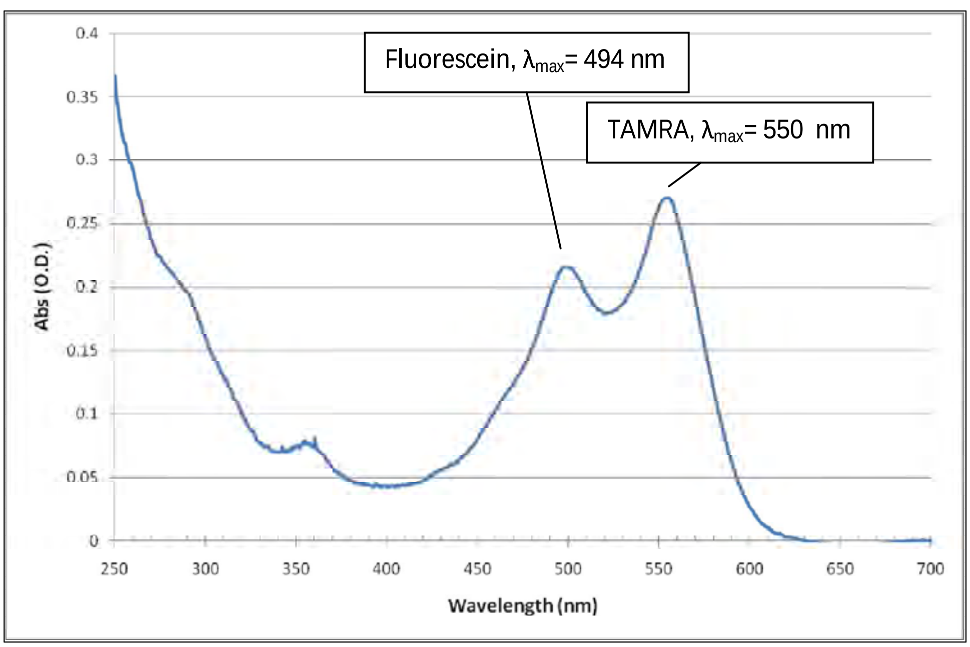


Figure 2: UV/Vis absorbance of product of labeling peptide RPLG*LWARC ("Broad2") with tetramethylrhodamine(TMR)-succinimidyl ester and fluorescein-maleimide dye derivatives.

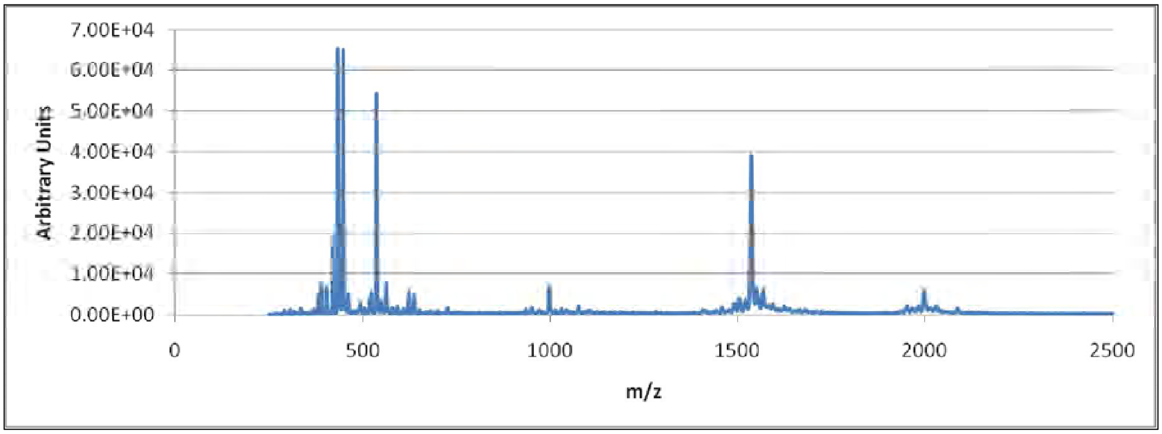


Figure 3: MALDI-MS data for reaction mix composed of reactants: Broad2 peptide (MW=1071), TMR-succinimidyl ester (MW=527.5) and fluorescein-maleimide (MW=427.4).

Furthermore, we observed cleavage of the substrate peptide by proteolytic enzymes (including several MMPs) and the consequent disruption of FRET quenching which yielded an enhancement in the fluorescence of the donating (sensor) fluorophore (Figs. 4,5).

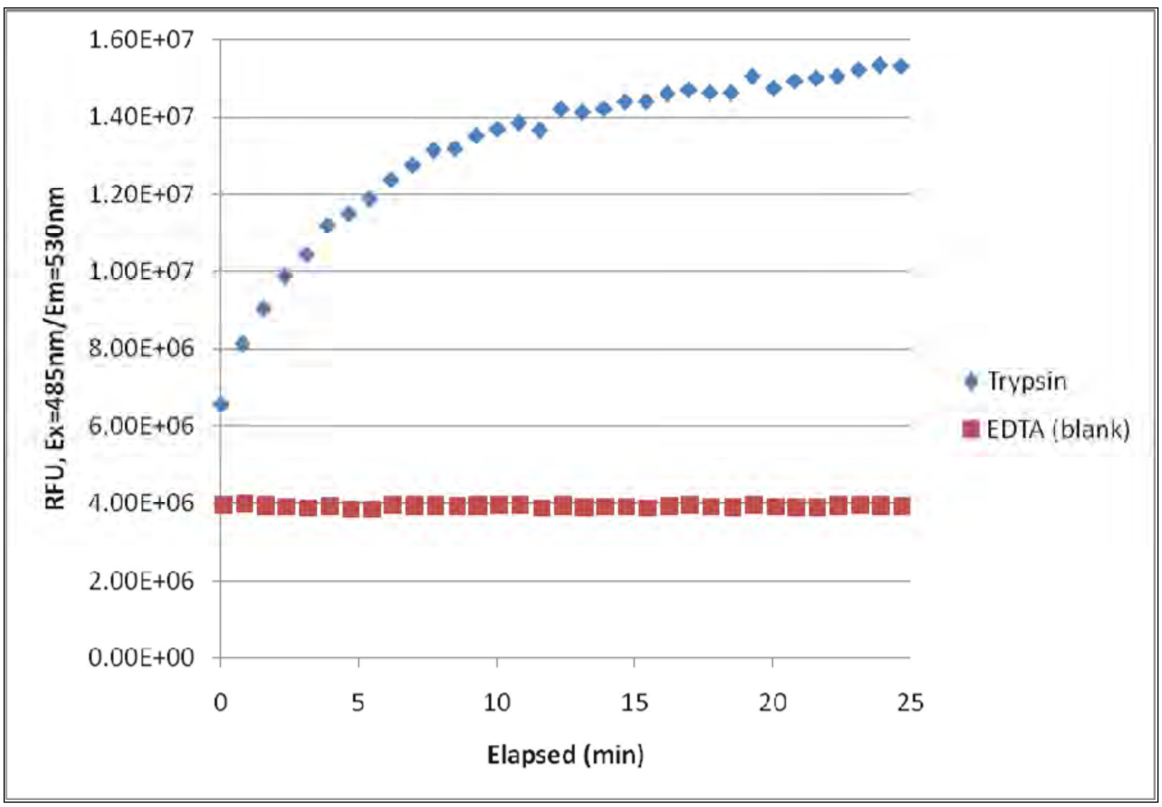


Figure 4: Fluorescein fluorescence over time upon treatment of FRET-labeled Broad2 peptide with trypsin or EDTA.

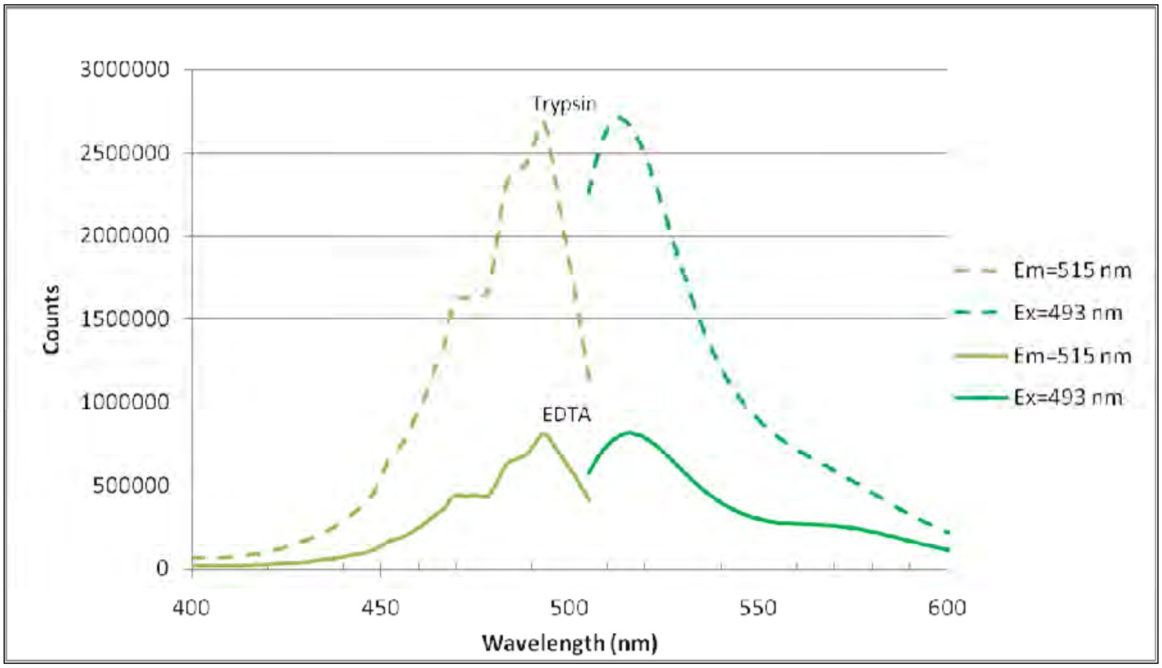


Figure 5: Fluorescence of FRET-labeled Broad2 peptide upon treatment with trypsin or EDTA. Cleavage by trypsin results in a dramatic increase in stimulated emission with no significant shift in peak excitation or emission.

These findings confirm that we can chemically synthesize cleavable, dye-conjugated substrate peptides that display both the desired FRET behavior when intact and the disruption thereof when enzymatically cleaved. Such structures represent the essential prerequisites for PNBs and PNTs.

b) Substrate selectivity: Incubate PNBs with MT1-MMP (MMP14) vs. other proteinases in the presence and absence of inhibitors (MMPIs, EDTA as a chelating agent) in cell-free systems. Determine selectivity of substrate peptide cleavage by analytical chemistry: fluorescence spectroscopy, HPLC, DLS. The best MT1-MMP substrate peptide for further syntheses will be indentified herein.

The proposed research aims to develop PNBs and PNTs that would be selectively cleaved by MMP14, a membrane-bound member of the MMP superfamily that is implicated in tumor cell invasion. Therefore we developed methods to test the selectivity of candidate substrate peptides for cleavage by various MMP family members. Briefly, candidate peptides conjugated with FRET-paired fluorophores were incubated with known concentrations of several MMPs in parallel while the fluorescence of the donor (sensor) fluorophore was monitored in real time as an indication of enzymatic cleavage. The efficiency of enzymatic cleavage of the peptide by an MMP was calculated as follows:

$$\frac{k_{cat}}{K_m} = \frac{M}{F_e C_e},$$

where M is the initial rate of fluorescence increase, F_e is the maximum fluorescence increase for a particular enzyme-substrate pair and C_e is the concentration of active enzyme (Fig. 6).

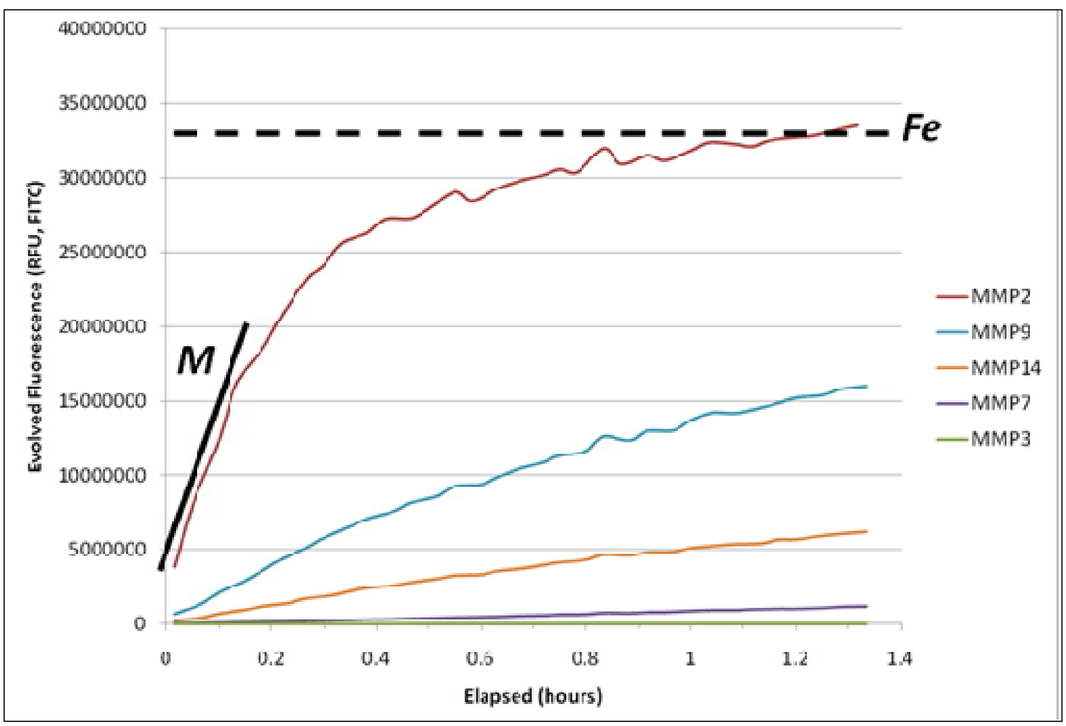


Figure 6: FRET-equipped MMP substrate peptide treated with MMPs 2, 3, 7, 9 and 14. Fluorescence of the FRET donor is tracked over time to allow calculation of initial rates of enzymatic cleavage.

While M and F_e were directly observable from this assay, C_e had to be determined separately. For this determination I performed an active site titration assay by incubating MMPs with serially diluted GM6001, a broad-spectrum tight-binding inhibitor of MMPs. Active MMP concentrations were then calculated by fitting data to the Morrison equation:

$$V = SA\left([E_0] - \frac{1}{2}\left(\left([E_0] + [I] + K_{i,app}\right) - \sqrt{\left([E_0] + [I] + K_{i,app}\right)^2 - 4[E_0][I]}\right)\right)$$

where V is the observed rate of fluorescence increase, SA is the specific activity of the MMP, $[E_0]$ is the active MMP concentration, $[I]$ is the GM6001 concentration and $K_{i,app}$ is the apparent inhibition constant.

Despite significant efforts, we have not yet identified a peptide that is selectively and efficiently cleaved by MMP14 after conjugation with fluorescent dye molecules. We hypothesized that steric hindrance caused by the attachment of relatively bulky dye molecules obscure a peptide’s cleavage site from the deeper binding pocket of some MMPs. Thereupon we examined four variants of a published MMP14 substrate sequence, altering the amino acid sequence length, terminal flexibility and size of attached fluorescent species (see Supporting Data).

Our findings support the conclusion that MMPs with relatively deep binding pockets may be unable to recognize short substrate sequences attached to relatively large dye molecules. Nonetheless, proof-of-principle experiments may be performed with substrates that are more broadly applicable to the MMP superfamily, as demonstrated in the fluorescence spectroscopy experiments previously discussed. Therefore we propose to continue with the development of nanoparticles that incorporate such peptides in parallel with ongoing experiments to develop an MMP14-selective substrate.

c) PNTs: Attach paclitaxel to N-terminal protected lysine residue of MT1-MMP-selective substrate peptide and attach entire complex as well as TAMRA and PEG-1000 to PAMAM-3 dendrimer using established chemistries. Validate synthesis with analytical chemistry techniques: UV-vis and fluorescence spectroscopy, HPLC, MALDI-TOF-MS, ninhydrin assay, dynamic light scattering (DLS).

To address selective delivery of therapeutic agents to breast tumor cells, we proposed to use established linking chemistry techniques to attach paclitaxel to our nanoparticles. My colleagues in the Matrisian laboratory employed these techniques to incorporate paclitaxel into Frechet dendron-based nanoparticles (nanodendrons) and compared the results of treatment with these nanoparticles versus the currently used clinical formation of albumin-bound paclitaxel known as Abraxane both in vitro and in vivo [1]. My colleagues' findings suggest that derivatized paclitaxel bound to nanoparticles may constitute an activatable pro-drug that can be more effective than Abraxane against breast tumor cells while reducing nonspecific injury to non-MMP-expressing cells, thereby reducing the side effects of treatment. The results also serve as proof-of-principle that PNTs can be activated by proteolytic cleavage in vitro, lending confidence to our ability to similarly incorporate paclitaxel into PNTs using an MMP14-selective substrate as yet to be determined.

d) In vitro Proteolysis: Incubate PNBs, PNTs and non-cleavable control dendrimers with available 17L3C and MDA-MB-231 cells. Determine in vitro proteolysis using fluorescence and confocal microscopy.

While my colleagues' results with nanoparticle-bound paclitaxel demonstrate that PNTs can be activated by proteolytic cleavage in vitro, I developed another method by which to test the potential for activation of PNBs. Briefly, I modified an established method for detecting invadopodia formation by cultured cells using fluorescently-labeled matrix proteins [2] to include a substratum of fluorescently-labeled MMP-selective substrate peptides (Fig. 7).

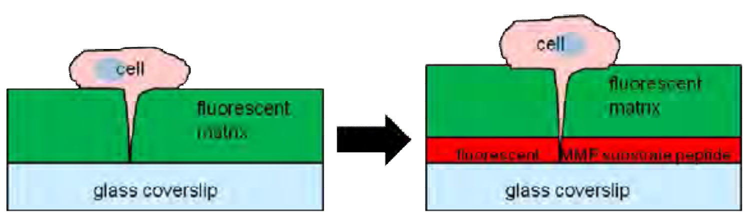


Figure 7: Schema representing modification of the established invadopodia assay to include a fluorescent MMP substrate peptide.

This modified invadopodia assay assesses by fluorescence microscopy whether or not cultured cells degrade the support matrix and subsequently the substrate peptide. In the case that the fluorescence intensities associated with both the matrix and the substrate peptide are deleted in co-localized puncta, one may qualitatively conclude that cells degrade matrix and possess active enzymes that are capable of cleaving the substrate (Fig. 8). If it is found that the matrix-associated fluorescence is deleted but the peptide-associated fluorescence remains relatively uniform, then one may infer that the cells degrade matrix but do not use enzymes capable of cleaving the substrate peptide in the process.

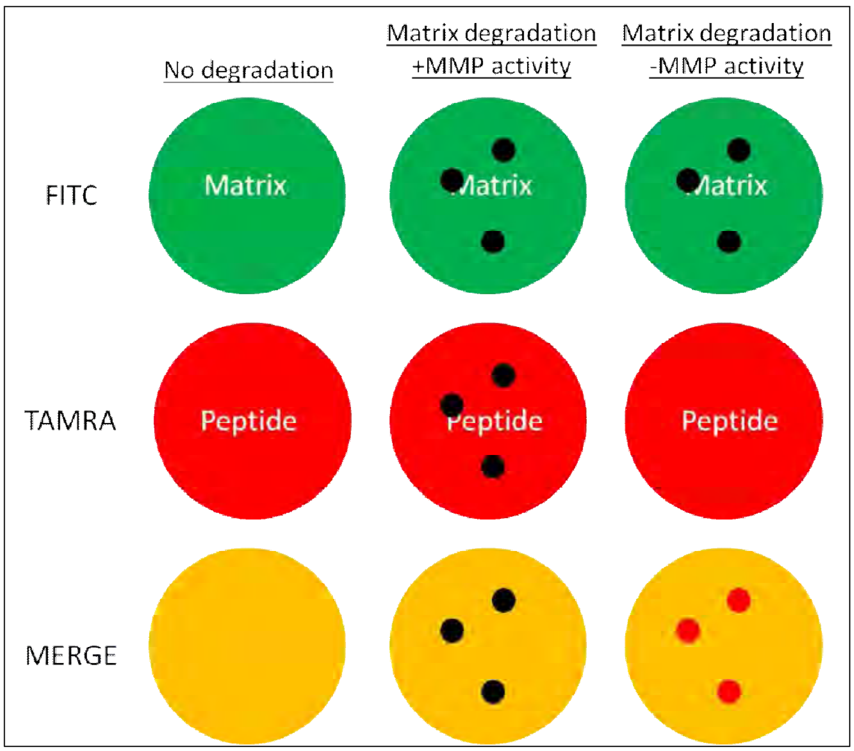


Figure 8: Expected potential outcomes of modified invadopodia assay.

For the purpose of establishing the assay, fluorescence microscopy results from MCF10A-CA1d human breast cancer cells cultured in this method suggest that many cells were capable of degrading the gelatin layer as well as the broadly MMP-selective “Broad2” peptide, confirming that MMP activity has a role in matrix-degrading invasive structures made by invasive breast cancer cells (Fig. 9).

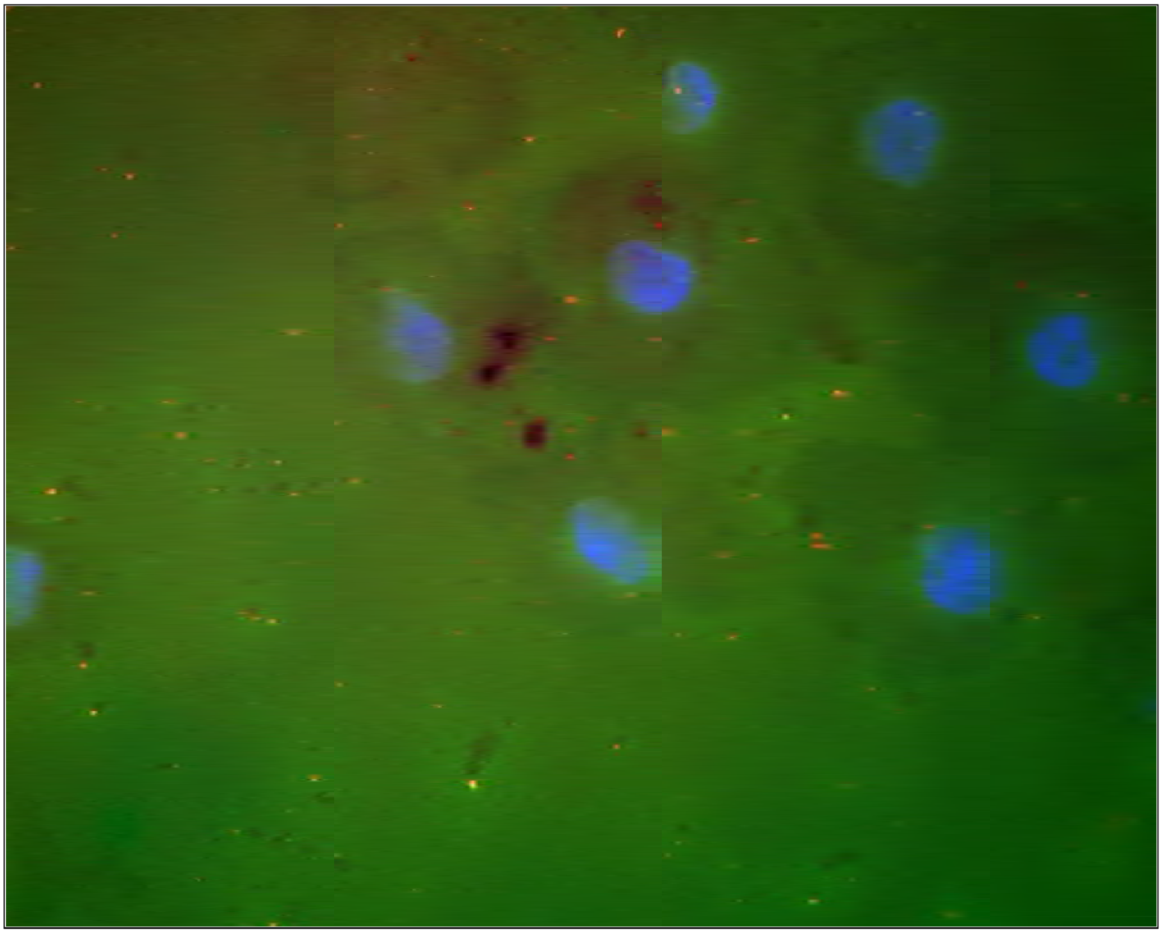


Figure 9: Fluorescence microscopy of modified invadopodia assay of MCF10A-CA1d human breast cancer cells with broadly selective MMP substrate "Broad2." Pseudo-color overlaid image represents signal intensity of Hoechst stain, blue; FITC-fibronectin, green; TAMRA-peptide, red.

Future experiments to validate PNB cleavability in vitro will include various mouse and human breast cancer cells (such as 17L3C and MDA-MB-231) as well as the use of substrate peptides conjugated with FRET-paired fluorescent dyes to allow semi-quantitative analysis. We will test PNB nanoparticles in cell culture media with various cell lines, inhibitors and conditions to determine the effects on MMP-specific activation. As discussed above, we propose to conduct these studies with a broadly selective substrate for proof-of-principle in parallel with the development of an MMP14-selective substrate for application in vivo.

KEY RESEARCH ACCOMPLISHMENTS

- Confirmed synthesis and proteolytic cleavage of FRET peptide MMP substrates
- Developed methodology for comparison of substrate selectivity
- Observed effective prodrug activity of conjugated paclitaxel in vitro and in vivo
- Developed assay for in vitro proteolysis of PNBs and observed activation of broadly selective MMP substrate

REPORTABLE OUTCOMES

The methods and findings discussed in this progress report have been presented at:

- The Vanderbilt Ingram Cancer Center and Department of Cancer Biology Departmental Seminar Series: Science Hour in Nashville, TN (Apr 2010)
- The 11th Annual Vanderbilt University Nanoscience and Nanotechnology Forum in Nashville, TN (Oct 2010)
- The 10th Annual Vanderbilt Host-Tumor Interactions Program and Department of Cancer Biology Joint Retreat in Lake Barkley, KY (Nov 2010)
- American Association of Cancer Research conference, Nano in Cancer: Linking Chemistry, Biology and In Vivo Applications in Miami, FL (Jan 2011)

CONCLUSION

The findings reported herein demonstrate that we have the ability to synthesize and characterize the key components of PNBs and PNTs. Furthermore, we have verified enzymatic cleavability and of substrate peptides and established methods by which to assess MMP selectivity. Evidence from in vitro and in vivo experiments suggests that the proposed strategy for MMP-activated selective delivery of diagnostic and therapeutic reagents is feasible.

While an MMP14-selective FRET substrate has yet to be identified, proof-of-principle has been demonstrated with other MMP-selective substrates. As such, we propose to proceed with the development of PNBs and PNTs with broadly MMP-selective substrates in parallel with screening for one appropriate for MMP14. With the synthetic and other experimental techniques established, substitution of an ideal substrate is straightforward.

Currently available therapies for breast cancer are limited by nonspecificity and system toxicities. Nanotechnology-based approaches offer an elegant means to overcome these limitations by multiplexing functional chemistries for medical image contrast and therapy with moieties for enhanced solubility and drug delivery. The dually-targeted, multifunctional nanoparticles we are developing are designed to selectively target and effectively diagnose or treat breast tumors, limiting damage to normal tissue and reducing side effects associated with traditional cancer therapies. The ultimate goal of this research is to provide a means to improve both the survival and the quality of life of people diagnosed with breast cancer.

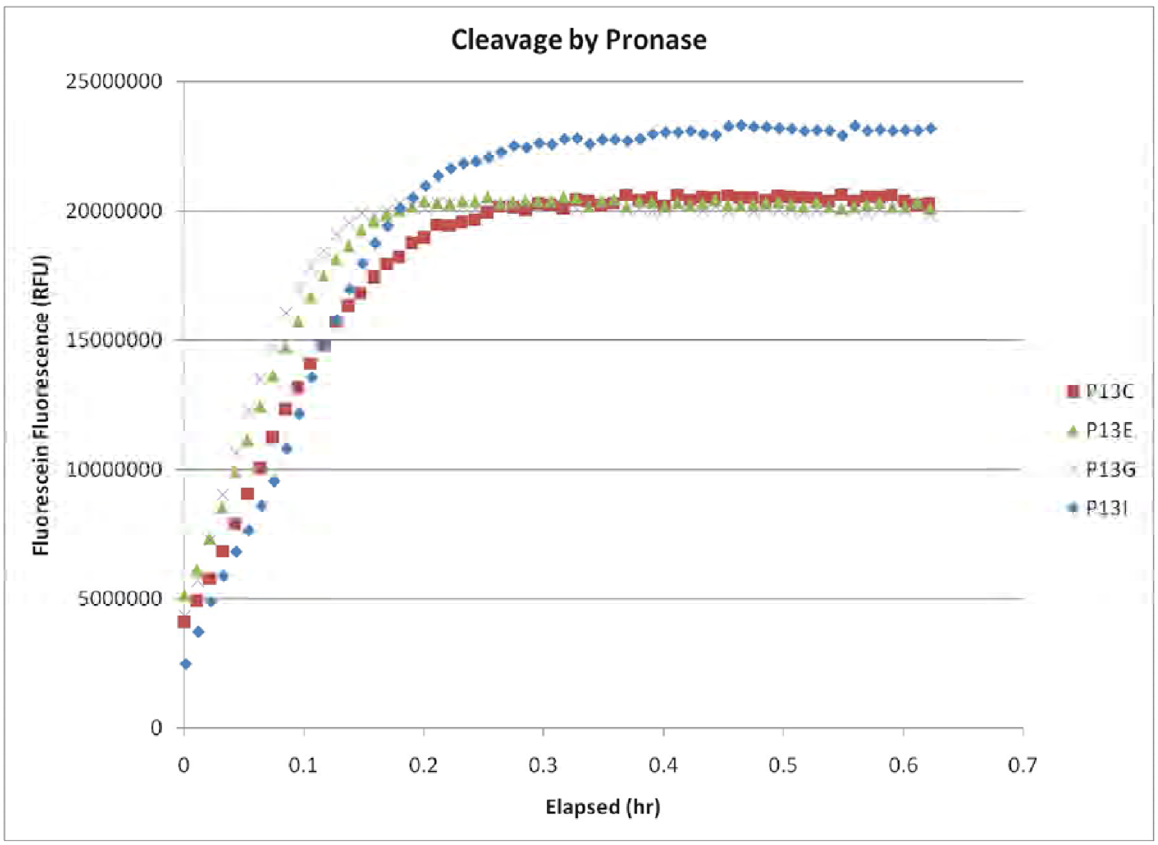
REFERENCES

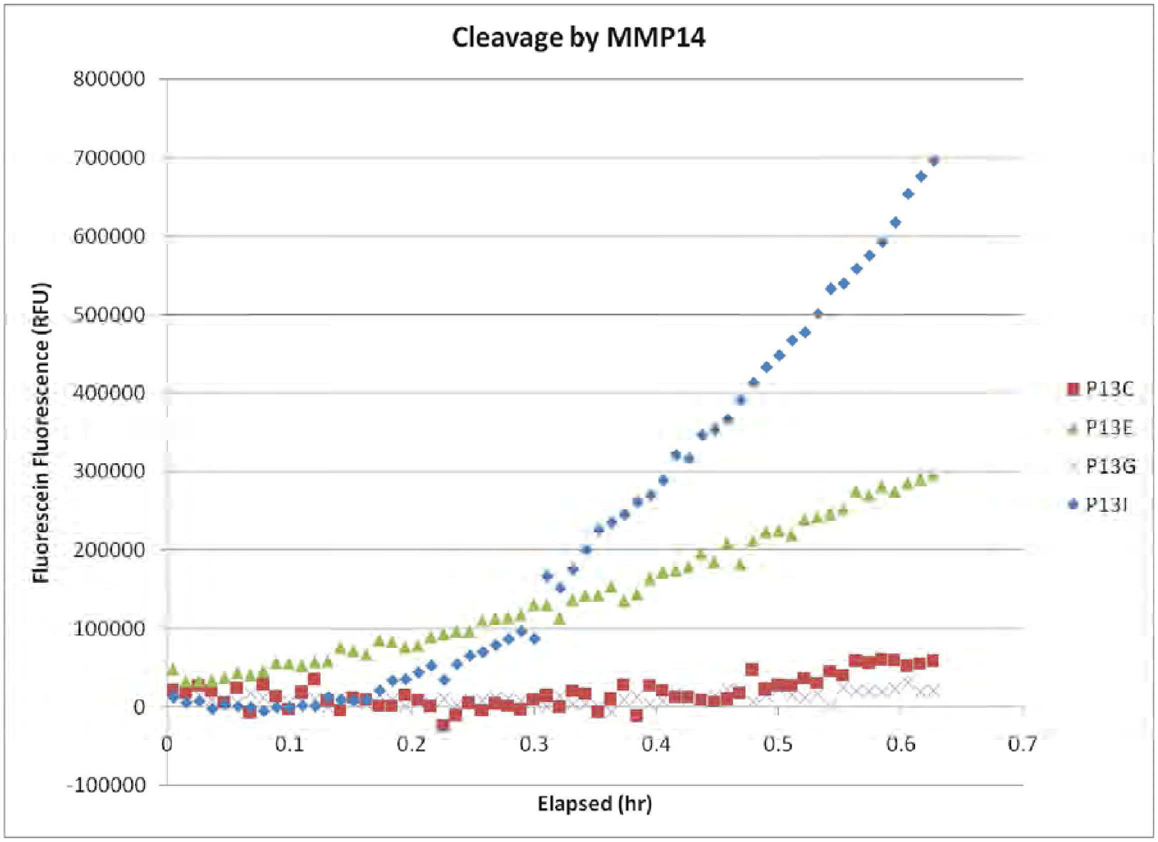
1. Samuelson, L., et al. *Nanodendrons for Imaging and Drug Delivery Targeted to the Tumor MicroEnvironment*. Manuscript in process.
2. Chen, W., et al (1994). *An in vitro cell invasion assay: Determination of cell surface proteolytic activity that degrades extracellular matrix*. Journal of Tissue Culture Methods 16(3):177-181.

SUPPORTING DATA:

MMP14 Substrate Peptide Steric Hindrance Experiment

Peptide Name	Sequence
Peptide #13c	TMR-RIGFLRTRK(FITC)R-cooh
Peptide #13e	TMR-SGRIGFLRTAK(FITC)R-conh$_2$
Peptide #13g	TMR-Ahx-SGRIGFLRTAK(FITC)R-conh$_2$
Peptide #13i	Dab-SGRIGFLRTAK(FITC)R-conh$_2$

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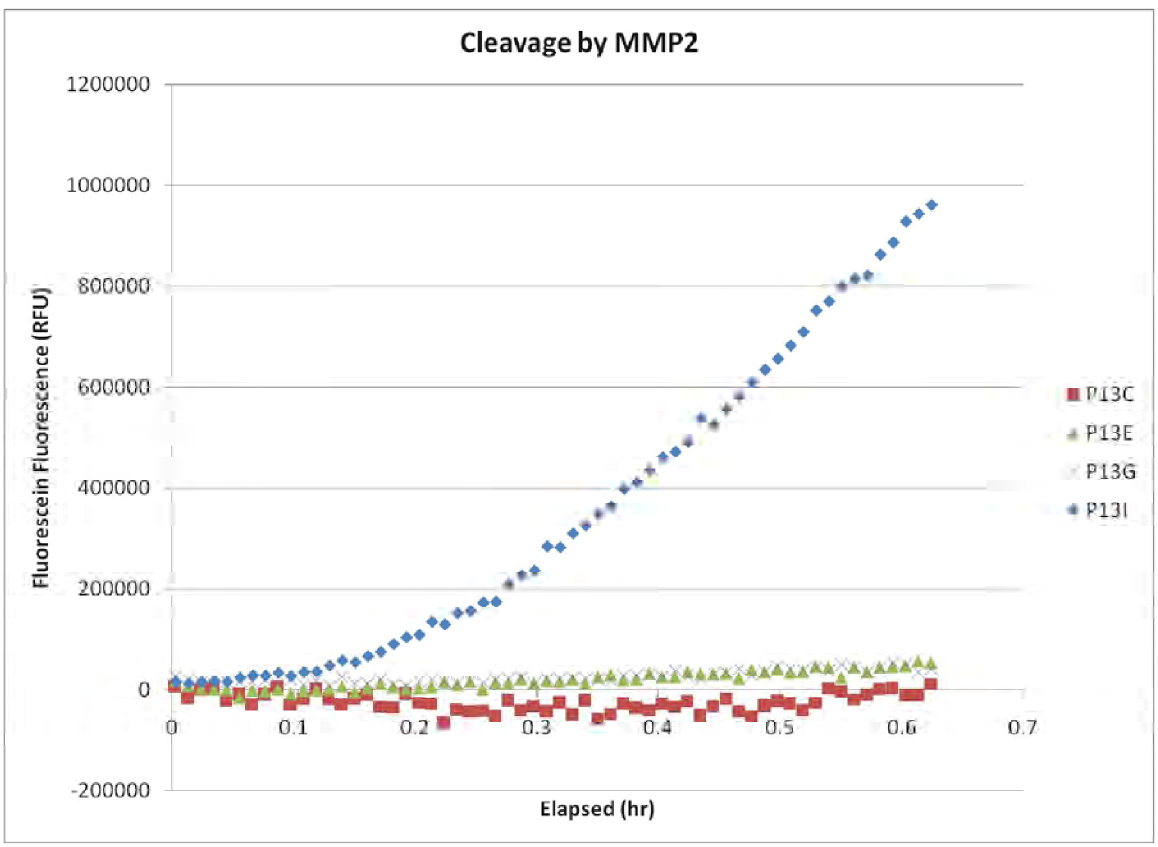
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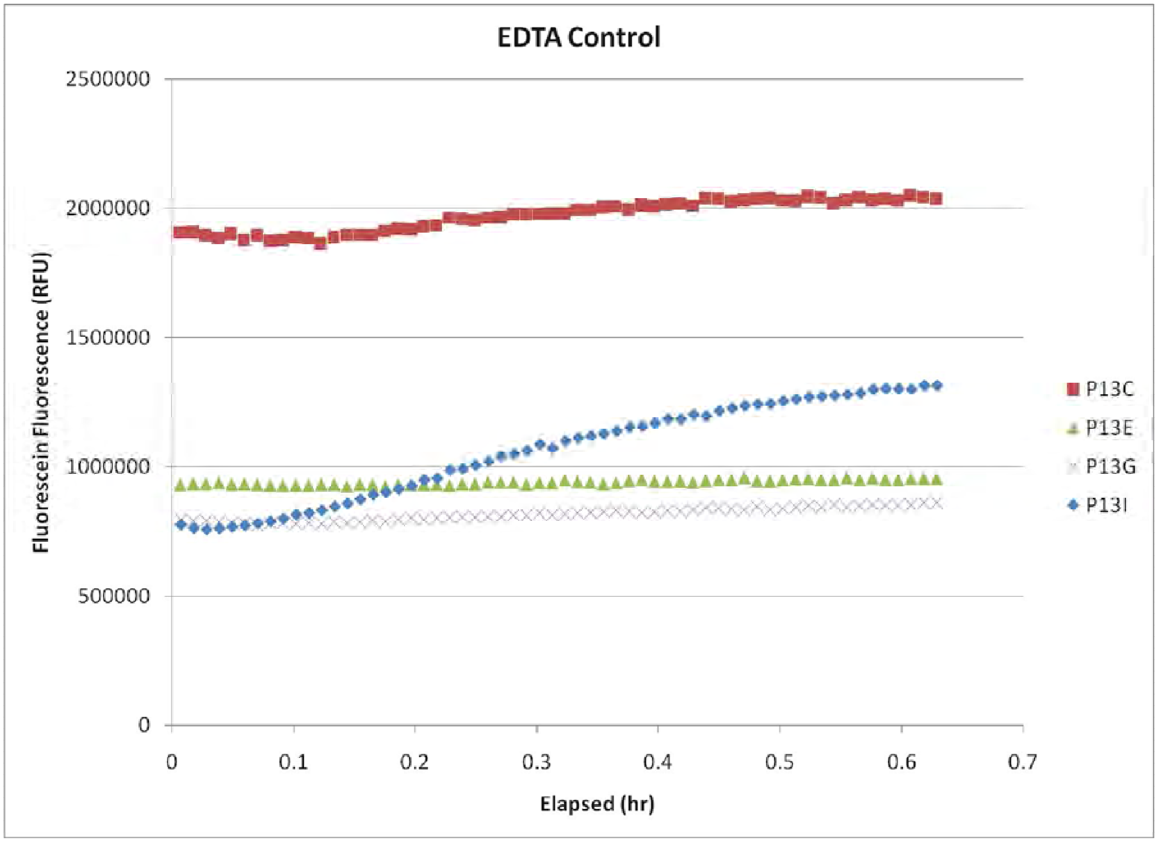
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